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Pheromone Deposition on Leaf Territories by Male Caribbean Fruit Flies, *Anastrepha suspensa* (Loew) (Diptera: Tephritidae)

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Male Caribbean fruit flies apply everted anal membranes associated with pheromone glands to the substrate while on leaf territories. There is a peak in abdominal dipping at the onset of the photoperiod, followed by a decline and then a more extensive period of activity in the second half of the photoperiod. During peak signaling periods, most males had a distinct set of lateral abdominal pheromone glands protruded for most of the time. However, the frequency of anal pheromone gland dipping varied considerably over time. Episodes of wing fanning (which may disperse pheromones) and abdomen dipping coincide during a male's tenure on a host leaf. Host-plant leaves were contained for 48 h with signaling males or immature females or kept without insects. Several, though not all, of the constituents of the pheromone were found on leaves within 1 h of removing signaling males. None of these compounds occurred on either type of control leaf. Mature virgin female flies were more likely to be found in contact with host leaves that had been previously exposed for 48 h to the activities of sexually mature males than with unexposed control leaves. There was no difference in the reaction of mature virgin female flies to unexposed control leaves or leaves previously exposed to the activities of other sexually immature female or male flies for 48 h. Presumably, the deposition of pheromones by mature males accounts for the difference in reactions.

KEY WORDS: Tephritidae; fruit fly; *Anastrepha*; pheromone; territory; lek.

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INTRODUCTION

Sexually active male Caribbean fruit flies occupy the underside of host plant leaves and defend these sites from rival conspecific males (Burk, 1983). While on their leaf territories, they broadcast pheromones, acoustic signals, and perhaps visual signals (citations by Sivinski and Burk, 1989; Sivinski and Webb, 1986). Male activities during these periods include a dipping motion that brings the abdominal tip to the leaf surface. The abdominal tip bears anal membranes that are everted during times of pheromone emission and appear as a pearly droplet (Nation, 1989). It has been suggested that the sheen on the membranes is a pheromone and that the membranes are evaporative surfaces that enhance dispersal of the chemicals (Nation, 1989). If so, dipping male fruit flies may be depositing pheromones on their territory (Nation, 1989) to increase evaporation, place markers that could orient female visitors, or play some other role in interactions with potential mates or sexual rivals.

In this paper, first, we measure the frequency of abdominal dipping with time of day and look at its relationship to wing fanning, a behavior that is known to produce acoustic sexual signals but that might also be involved in pheromone dispersion (Sivinski *et al.*, 1984). Second, we demonstrate that abdominal dipping deposits pheromone components on leaves. Third, we show that pheromones deposited on leaves attract and/or arrest the motion of mature virgin female Caribbean fruit flies and, so, potentially play a role in the species' sexual behavior.

MATERIALS AND METHODS

Caribbean fruit flies were obtained from a colony maintained by the Florida Division of Plant Industry in Gainesville. Its stock has been under domestication for more than 15 years. The flies were kept on a 12L:12D (0600–1800 h, light) photoperiod at about 25°C. Voucher specimens are in the collection of the authors.

Observations on the diel pattern of abdominal dipping were carried out under room lighting (6.8 lux at cage surface) and room temperature (about 25°C) and humidity. Ten sexually mature virgin males (10–15 days old) were held in a 15 × 15 × 15-cm Plexiglass cage. A 15-cm-diameter piece of filter paper was held to the ceiling by magnets inside the outside of the cage to provide shelter conducive to sexual displays. Five such cages were set up the afternoon prior to observation and the flies were provided with water and food consisting of a yeast hydrolysate and sugar mixture. The following day, each cage of flies was observed hourly for a period of 2 min during the entire 12-h photoperiod. During the observation period, the number of flies with lateral pheromone glands protruding was counted, as was the number of times their abdominal tips touched

the substrate and the number of "pulse trains" [bursts of wing fanning that produce the "calling song" (see Burk and Webb, 1983; Webb *et al.*, 1983)]. The observations were repeated three times so that the activities of 150 males in 15 cages were sampled. Means were compared through analysis of variance and Waller Duncan *k*-ratio *t* test (SAS Institute, 1987).

Observations of the timing of dipping and wing fanning were carried out under seminatural conditions. A small host tree (guava; *Psidium gujava* L.) about 2–2.5 m high was placed in an outdoor field cage. A mesh bag 66 cm long and sealed with Velcro was placed over a branch with 12–24 leaves. Six mature virgin males (10–15 days old) were placed in the bag. Continuous observations of flies occupying a particular leaf during the afternoon peak (i.e., 1400–1800 h) of sexual activity were begun immediately and continued uninterrupted as long as clear observations and the identity of the flies could be maintained. Observations of acoustic production and dipping were recorded on a cassette recorder, and the activities and their timing quantified later. Twenty-one flies on 17 leaves were observed for their total tenure on a leaf. Total observation time was 5 h.

The following bioassay was used to determine the reaction of mature virgin female flies to leaves that had been exposed to sexually active males. Two sexually mature virgin males were placed in a 450-ml plastic cup. The central portion of the lid was removed and replaced by, first, a host-plant leaf [loquat *Eriobotrys japonica* (Thumb) Lindl] and, then, a piece of fine mesh cloth. These were held in place by the rim of the lid. The result was that the ceiling consisted largely of host-plant leaf. Sugar and yeast hydrolysate were provided as food. Water was provided by a wick that protruded from the floor of the cup and whose other end was submerged in water held by another 450-ml cup directly under the fly cage. Males and leaves were kept together for 48 h so that the occurrence of at least one episode of pheromone emission was likely. Control leaves were placed on cages containing food and water but no flies and were likewise held for 48 h before use. After 2 days, two exposed and two control leaves were immediately placed in a 25 × 25 × 25-cm Plexiglas and fabric mesh cage. These were held to the ceiling by magnets. Thirty to forty virgin females were released into the cage and allowed to adjust to their surroundings for 5 min. After that period, the number of females contacting the leaves was counted every 5 min for the next half-hour. To control for the possibility of certain positions being more attractive regardless of the type of leaf placed there, all bioassays were run in pairs with a fresh set of leaves and flies placed into a fresh cage, but with the leaf position changed. Fresh materials were used to avoid the deposition by flies of possibly attractive substances such as feces or sugar–saliva drops on a leaf or cage surface that might result in attraction to spots on the cage. The paired set of observations was considered a single replicate. Data were calculated as the mean proportions of flies that were found on

exposed leaves. Means were arcsine transformed and compared by *t* test (SAS Institute, 1987). Seventeen replicates were performed. Because of the possibility that flies were leaving attractive substances other than pheromones on leaves, such as sugar–saliva droplets or bacteria from feces, other bioassays were performed using leaves kept for 48 h with sexually immature females and males (1–3 days old) rather than sexually mature males. The males in this experiment were wild flies obtained from various host fruit in Monroe County, Florida. Procedures were identical and eight replicates each of males and females were performed. To determine if leaves used in the experiment were of similar size and, thus, equally likely to shelter randomly distributed insects, 36 control and 35 exposed leaves were chosen at random and dried under vacuum at 80°C for 72 h. These were weighed and their mean weights compared by *t* test (SAS Institute, 1987).

To demonstrate that pheromones persist on male leaf territories and that, when present, their deposition is due to abdominal tip touching and not an accumulation of volatiles, 12 pairs of sexually mature males were caged with loquat leaves in the previously described manner. In another 12 cages, the rim and top 2 cm of a 450-ml plastic cup were placed on top of the cloth mesh and inserted into the male-holding cup. This lowered the cloth ceiling by about 2 cm. The loquat leaf was then placed above the cloth and out of contact with flies. After 48 h, leaves were sorted by treatment and placed in glass chambers (four leaves per chamber) for immediate collection of volatiles. For comparative purposes, additional collections were made from three empty glass chambers that had held five sexually mature males for 36 h. Procedures developed for collections of volatiles from calling Caribbean fruit fly males were used (Heath and Manukian, 1993). Briefly, volatiles were collected onto filters using Super-Q (Altech Associates Inc., Deerfield, IL) adsorbent by drawing 1 liter/min of purified air through the chambers for 2 h. Filters were eluted with 200 μl of methylene chloride, and 100 ng of *n*-tetradecane was added as internal standard for subsequent analyses. Volatiles were analyzed by capillary gas chromatography using a retention gap column prior to the analytical capillary column. The retention gap column used was a 10 m × 0.25-mm-ID trimethylsilane deactivated fused silica (Quadrex, New Haven, CT) and the analytical column was a 50 m × 0.25-mm ID BP-1 (apolar phase). Gas chromatographic analyses were conducted using a Hewlett–Packard Model 5890 gas chromatograph, equipped with cool-on column capillary injector (septum injector) and flame ionization detector. Helium was used as the carrier gas at a linear flow of 18 cm/s and the temperature program was isothermal at 60°C for 2 min, then temperature programmed at 20°/min to 180°C. The chromatographic data were stored and analyzed in a Nelson 4000 data system.

Mass spectra were obtained to confirm the known pheromonal compounds and to identify new compounds using the BP-1 capillary column, operated as

described above, coupled to a Finnigan Ion Trap mass spectrometer in either the electron impact (EI-IDTMS) or the chemical ionization (CI-ITDMS) mode. The reagent gas used for CI was isobutane.

RESULTS

The diel pattern of abdominal dipping follows that of male lateral gland protrusion and acoustic signaling (see Hendrichs, 1986; Nation, 1990; Landolt and Sivinski, 1992). There is a peak immediately after the onset of the photoperiod, followed by a decline and then a more extensive period of activity in the second half of the photoperiod (Fig. 1). However, these early and late signaling periods are different in the relative frequency of dipping and pulse train production. Wing fanning is rare relative to dipping during the morning signaling period ($F = 6.62$, df 9, $P = 0.0001$) (Fig. 2).

Episodes of wing fanning and abdomen dipping coincide during a male's tenure on a host leaf (Fig. 3). While the two behaviors occur together, they are not always performed at an equal intensity ($r = 0.53$).

Mature virgin female flies were more likely to be found in contact with host leaves that had been previously exposed for 48 h to the activities of sexually mature males than with control leaves [mean proportion of time on exposed leaf = 0.62 (0.02), control mean = 0.38 (0.02); $t = 8.8$, df = 32, $P < 0.0001$]. There was no difference in the reaction of mature virgin female flies to control leaves or leaves previously exposed to the activities of sexually immature female flies for 48 h [mean proportion of time on exposed leaf = 0.51 (0.02), control

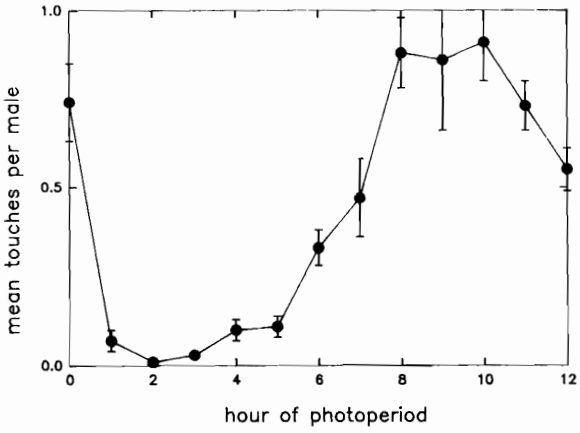

Fig. 1. Mean (SE) touches of the abdominal tip to the substrate during 2-min observations made at hourly periods during the photophase; $n = 15$ cages of 10 males each.

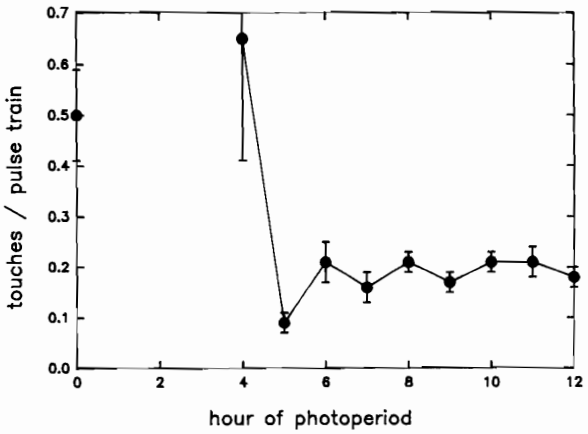

Fig. 2. The ratio of abdominal tip touches to the substrate to pulse trains produced (bursts of wing fanning). Hours 1, 2, and 3 were excluded because of low signal production (see Fig. 1).

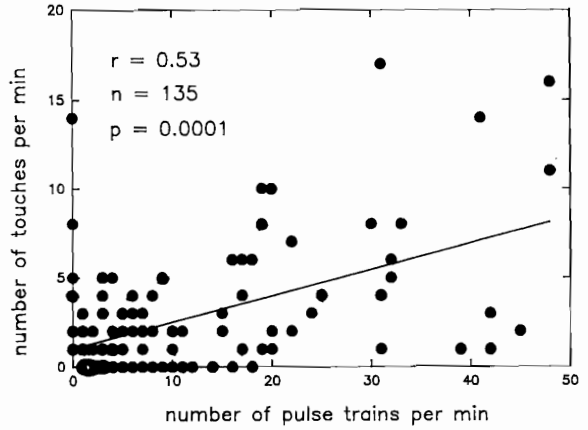

Fig. 3. The number of abdominal touches to the substrate in a minute of observation time correlated with the number of pulse trains in a minute of observation time. Only observation minutes during which one or the other behavior occurred were included in the analysis.

mean = 0.49 (0.02); $t = 0.9$, df = 14, $P = 0.40$]. Nor was there a preference toward leaves exposed to immature males [exposed mean = 0.48 (0.03), control mean = 0.52 (0.03); $t = 1.14$, df = 14, $P = 0.27$]. Presumably, the deposition of pheromones by mature males accounts for the difference in reactions. There

was no difference in the dry weight of control and exposed leaves [exposed = 12.3 g (0.05 g), control = 12.9 g (0.06 g); df = 79, $P = 0.29$].

Five of eight putative Caribbean fruit fly pheromone components were recovered from loquat leaves that were in direct contact with calling males and from the empty chamber that had previously held calling males. These were suspensolide (TR, 14.2 min), *E*,*E*-α-farnesene (RT, 14.6 min), β-bisabolene (RT, 14.7 min), anastrephin (RT, 15.4 min), and epianastrephin (RT, 15.7 min). Except for the lack of recovery of ocimen (RT, 7.7 min), (*Z*)-3-nonenol, and (*Z*,*Z*)-3,6-nonadienol (RT, 9.2 min), the components obtained from the empty chamber were at the expected ratios (Nation, 1990). An exception was an unexpectedly large peak at the retention time for farnesene. This was the only peak that was obtained from the loquat leaves that were placed above, but not in contact with, calling males. Nation (1990) found that 6–8% of the total anastrephin and epianastrephin produced by male Caribbean fruit flies remained on the walls of his volatile collection vessels but did not recover the other compounds presently found on leaves.

DISCUSSION

Deposition of chemical signals on territories is common in male mammals ranging from mice to hippopotomi (Sebeok, 1977). Such chemicals are directly or indirectly important to male mating success; i.e., they either attract potential mates or help maintain territories that are critical in obtaining mates. Similar behaviors have been described less frequently in insects and most of these cases occur in the Hymenoptera. Certain bees, particularly bumblebees, mark various plants on long patrol routes with a mandibular gland substance (e.g., Svensson, 1980). Females will wait on these markers until the male arrives (van Honk *et al.*, 1978). This type of scent marking is also performed by the polistine wasp *Mischocyttarus labiatus* (Litte, 1981). Males of the pteromalid parasitoid *Nasonia vitripennis* mark spots where they have copulated in order to organize their foraging for mates in sexually productive areas (van dem Assem *et al.*, 1980).

Among the Diptera, pheromone deposition on leaf territories have been hypothesized to occur in both a number of *anastrepha* spp. (M. Aluha, personal communication) and the Mediterranean fruit fly, *Ceratitis capitata* (Weid.) (Prokopy and Hendricks, 1979). The function of purported pheromone deposition in these flies is unknown, but since virgin female Caribbean fruit flies were more likely to be found associated with marked leaves in the laboratory, marking may be a means of amplifying an attractant by exposing volatiles over a greater surface area. Alternatively, the pheromone, or its less volatile components, may "arrest" females that contact it on male territories and better allow the male to address acoustic, visual, or chemical courtship signals to potential mates. Whatever its role, it is now clear that abdominal tip dipping does deposit pheromone

on leaf surfaces. There it or certain of its components remain long enough to be detected by immediate chemical analysis.

In the field, flies occasionally occupy particular leaves every afternoon over periods of days (Sivinski, 1989). Perhaps flies prefer leaves that have accumulated pheromone constituents and so are particularly effective as signaling sites. Males attempt to take over occupied leaves and residents defend their territories. Again, the presence of pheromone constituents may make leaves a resource valuable enough to fight over. On the other hand, leaf tenures are sometimes brief, perhaps due to changing light conditions (Sivinski, personal observation), and the relative importance of pheromone deposits compared to other environmental factors is unknown.

It is not clear why abdominal dipping is associated with wing fanning. Wing motions are known to produce an accurate signal that serves in both courtship and attraction (Sivinski *et al.*, 1984; Webb *et al.*, 1983). It is possible that wing fanning is also useful in disseminating pheromones from either leaf or body surfaces. If so, wing fanning's intermittent nature is puzzling. Could it be most useful when pheromones are freshly deposited? This raises the question of why pheromone deposition on leaves, but not lateral abdominal pheromone gland protrusion, is sporadic. Perhaps males cannot produce sufficient pheromones to apply them continually to their territories or, for some unknown reason, a leaf-based pheromone signal repeated at intervals is an effective and economical substitute for continuous emission (see Sivinski, 1980).

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